PATENT
19036/34546

IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE

In re Application of: Wakamiya, N.	CERTIFICATE OF MAILING BY EXPRESS MAIL
Serial No.: To Be Determined	"EXPRESS MAIL" mailing label No. EM099827381US
Filed: Herewith (US National Phase of PCT/JP96/00173, Filed 25 January 1996)	Date of Deposit: February 17, 1998
Title: "Recombinant Conglutinin and Producing Method Thereof"	I hereby certify that this paper and the documents referred to herein as enclosed herewith are being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. §1.10 on the date indicated above and are addressed to Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231.
Group Art Unit: To Be Determined	
Examiner: To Be Determined	Richard Zimmermann

PRELIMINARY AMENDMENT "A"

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Please amend the above-identified patent application as follows before calculating the filing fee and before examination on the merits.

Amendments

In the specification:

Please delete pages 19-21 of the translation of the specification filed herewith, which constitute a translation of the sequence listing, and substitute therefor new pages 19-23 appended hereto, which constitute a substitute Sequence Listing. Please renumber the pages of claims beginning with page number 24.

In the claims:

Please amend claims 2, 5, and 7 and add new claim 8 as shown below:

2. (Amended) A method for producing the recombinant conglutinin of claim 1 [comprising a native conglutinin fragment] comprising the steps of:

(a) preparing a vector inserted there into cDNA corresponding to 613 bp through 1113 bp of the native conglutinin DNA,

(b) obtaining transformants by introducing said vector into *Escherichia coli* JM109,

(c) incubating said transformants in an appropriate medium,

(d) infecting said incubated transformants with phage, and

(e) collecting recombinant conglutinin from the phage-infected transformants,

wherein said recombinant conglutinin comprises a collagen region having two units of amino acids sequence of Gly-Xaa-Xaa (SEQ ID NO. 3), a neck region of the native conglutinin and a carbohydrate recognition domain of the native conglutinin, and 2nd and 3rd amino acids in said amino acid sequence of Gly-Xaa-Xaa are protein-constituting amino acid.

5. (Amended) The method for detecting the anti-virus activity according to [the] claim 3 [or 4] wherein said virus is Influenza A virus.

7. (Amended) Human Mannan-binding protein (hMBP) according to claim 6 having anti-Influenza A virus activity.

-- 8. The method for detecting the anti-virus activity according to claim 4 wherein said virus is Influenza A virus. --

New Abstract of the disclosure:

Please amend the application by adding the attached Abstract of the Disclosure as page 26 of the translation of the application filed herewith, after the claims and prior to the drawing sheets.

Remarks

The sequences in the original and substitute Sequence Listings are identical. The substitute Sequence Listing has been prepared with the Patent Office's preferred PatentIn software and is accompanied by the requisite computer-readable copy and statement.

The amendments to the claims are merely intended to minimize the filing fee and are not intended to change the scope of the claims. The Applicant does not intend by these or any other amendments to abandon the subject matter of any claim as originally filed, and reserves the right to pursue such subject matter in this application or related applications, such as continuing applications.

The Abstract of the disclosure is identical to the abstract found on the cover of the published PCT application from which the present application is derived, and it finds support throughout the application.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN
6300 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6402

Date: February 17, 1998

David A. Gass
Reg. No. 38,153

PATENT
19036/34546A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Wakamiya	)	"EXPRESS MAIL" mailing label No. EK657816384US
Divisional of U.S. patent application Serial No.: 09/029,156	)	Date of Deposition: November 8, 2001
Serial No.: Not yet assigned	)	I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to: Commissioner for Patents, Box PCT, Washington, D.C. 20231
Filed: Herewith	)	
Title: Recombinant Conglutinin and Prducing Method Thereof	)	
Expected Group Art Unit: 1647	)	
Expected Examiner: R. Landsman	)	Richard Zimmerman

PRELIMINARY AMENDMENT "B"

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to examination on the merits, please enter the following preliminary amendment into the divisional application filed herewith. Preliminary Amendment "A" was filed as part of the parent application and was referred to in the declaration, and is accordingly considered part of the present application as well. A copy of Preliminary Amendment "A" is filed herewith.

AMENDMENTS

In the specification:

Please amend page 1 by adding as the first sentence of the application, immediately after the title, the following priority claim: --This application is a divisional application of U.S. patent application Serial No: 09/029,156, filed August 3, 1998, which is 35 U.S.C. §371 national filing of PCT/JP96/00173, filed January 25, 1996, which in turn is a

continuation-in-part of PCT/JP95/02035, filed 02 October 1995, now U.S. Patent No. 6,110,708--

Please delete the paragraph at page 2, lines 19-25, and substitute the following paragraph:

Similar methods had also been tried by using another expression vectors, but the same or less expression level had merely detected by any of the vectors. Anyway, an effective expression system have not yet been realized in the art. This seems due to difficulties in expressing the conglutinin because *Escherichia coli* does not possess proteins of the structure like collagen-like region. Further, yield of the conglutinin produced from an eukaryotic cells is little, and some of the conglutinin may sometimes have an inappropriate post-transcriptional modification.

Please delete the paragraph at page 4, lines 9-10, and substitute the following paragraph:

Figure 8 shows conglutination activities on the recombinant conglutinin and the native conglutinin with microtiter plate assay system;

Please delete the paragraph at page 5, lines 21-25, and substitute the following paragraph:

PCR products of Example 1(1) were digested with the restriction enzymes XhoI and EcoRI, then were inserted into the expression vector pRSET-A (Invitrogen) with DNA ligation kit (Takara Shuzo). Then, pRSET vector so prepared from pRSET-A containing bovine conglutinin cDNA fragment was transfected into *Escherichia coli* JM109 and transformants were obtained that have the conglutinin DNA fragments corresponding to 631 bp through 1113 bp of the native conglutinin DNA (Figure 1).

Please delete the paragraph at page 5, line 26, to page 6, line 2, and substitute the following paragraph:

Sequences of these fragments were corresponding to 191st through 351st amino acids of native conglutinin, namely, PCR exactly amplified the sequences having the short collagen region, the neck region and the carbohydrate recognition domain. Further, there was

no error in the PCR reaction. Accordingly, desirable stable transformants were obtained which can remarkably produce such conglutinin DNA fragments.

Please delete the paragraph at page 10, lines 1-9, and substitute the following paragraph:

After coating the microtiter plates with yeast mannan (1 μg/well), the recombinant conglutinins were reacted with sugars. Sugar binding specificity (I_{50}) was shown as sugar concentration to halve binding activities. Results are shown in Table 1. Obviously from Table 1, sugar binding activities with the recombinant conglutinin are substantially same to that of the native conglutinin. Then, as shown in Figures 6 and 7, like the native conglutinin, binding activities of the recombinant conglutinin were dependent on calcium ion. Further, these binding activities were inhibited by N-acetylglucosamine. On the other hand, tags of histidine fused to the recombinant conglutinin were not involved in the binding activities to mannan and binding specificities.

Please delete the paragraph at page 11, lines 25-30, and substitute the following paragraph:

Conglutination activities of the recombinant conglutinin and the native conglutinin were evaluated by Microtiter plate assay system. Sheep erythrocyte cells with iC3b were prepared according to the method of Wakamiya *et al.*, (*Biochem. Biophys. Res. Comm.*, Vol. 187, pp. 1270-1278, 1992). Namely, 1 % sheep erythrocyte cells with iC3b were prepared by priming with a mixture of ten-fold diluted fresh horse serum and equivalent amount of anti-Forssmann antibody, and incubated at 37 °C for ten minutes.

Please delete the paragraph at page 12, lines 1-10, and substitute the following paragraph:

50 μl of 1 % sheep erythrocyte cells with iC3b and 50 μl of the recombinant conglutinin or 50 μl of the native conglutinin were added to the raw veronal buffer or the veronal buffer containing 30 mM N-acetylglucosamine. Then, they were incubated at 37 °C and the conglutination activities thereon were detected. The lowest concentration of the proteins to cause agglutination is regarded as titer of conglutination, then the results are shown in Figure 8. In Figure 8, Lane A is the native conglutinin, Lane B is the recombinant

conglutinin and Lane C is the recombinant conglutinin containing 30 mM N-acetylglucosamine. Titer of conglutination on the native conglutinin was 0.16 μg/ml, while that of the recombinant conglutinin was 1.3-2.5 μg/ml. Such activities were completely inhibited by 30 mM N-acetylglucosamine (GlcNAc).

Please delete the paragraph at page 12, line 22, to page 13, line 4, and substitute the following paragraph:

In accordance with the method of Okuno *et al.*, (*J. Clin. Microbiol.*, Vol. 28, pp. 1308-1313, 1990), experiments were performed in 96-well microtiter plates with 1 % chick's erythrocytes. The ether-treated virus antigens from a hen egg antigen was used. No additive had been added to mixed cultivation solution of TBS/C (TBS solution containing 5 mM sodium chloride) except for 30 mM N-acetylglucosamine or 10 mM EDTA. After incubation at room temperature for one hour, effects on the recombinant conglutinin fragments (rBKg-CRD) against viral hemagglutination on chick's erythrocytes were observed. Results are shown in Table 2. Results on Influenza A virus A/Ibaraki/1/90 are shown in Figure 9. In Figure 9, Lane A is the native conglutinin, and Lanes B, C and D are directed to the recombinant conglutinin fragments, in which the Lane B is no additives, Lane C is added thereto 30 mM N-acetylglucosamine and Lane D is added threreto 10 mM EDTA.

Please delete the paragraph at page 13, lines 18-22, and substitute the following paragraph:

Hemagglutination Inhibition (HI) activities were depended on dosages and calcium. Further, Hemagglutination Inhibition (HI) activities of the recombinant conglutinin are substantially the same level to the titer of the native conglutinin, rat surfactant protein D, human surfactant protein D (Hartshorn *et al.*, *J. Clin. Invest.*, Vol. 94, pp. 311-319, 1994).

Please delete the paragraph at page 15, lines 19-25, and substitute the following paragraph:

Physiological activities against Influenza A viruses were evaluated in accordance with the evaluation method on Hemagglutination Inhibition (HI) Activities according to Example 5, the evaluation method on Neutralization Activities according to

Example 6, the evaluation on Hemagglutinin (HA) Activities by Western blotting, and the present method referred to in Example 7. Further, the neutralization activities against Influenza A viruses by the various collectins were also evaluated by the method referred to in Example 6. Results were shown in the following Tables 4 and 5.

In the claims:

Please cancel all claims, 1-8, and enter new claims 9-10 as follows:

9. A purified Mannan-binding protein (MBP) having budding inhibition activity as determined by a method comprising steps of:

(a) culturing cells in presence of virus(es) to prepare virus(es)-infected cells,

(b) presenting the virus(es)-infected cells in presence or absence of the MBP,

(c) comparing gross area of virus(es)-infected focus formed in the presence of the MBP with that formed in the absence of the MBP, and

(d) evaluating from the comparison results in the step (c) an inhibition level by the MBP on budding of virus in said infected cells.

10. A purified Human Mannan-binding protein (hMBP) having anti-Influenza A virus activity as determined by the method comprising the steps of:

(a) culturing cells in presence of virus(es) to prepare virus(es)-infected cells,

(b) presenting the virus(es)-infected cells in presence or absence of a calcium-dependent lectin,

(c) comparing gross area of virus(es)-infected focus formed in the presence of a calcium-dependent lectin with that formed in the absence of a calcium-dependent lectin, and

(d) evaluating from the comparison results in the step (c) an inhibition level by the calcium-dependent lectin on budding of virus in said infected cells,

wherein the calcium-dependent lectin comprises an N-terminal region containing cysteine, a collagen-like region, a neck region and a carbohydrate recognition domain.

REMARKS

The specification is amended to include a priority claim to the parent applications. All of the other amendments to the specification merely are directed to grammatical and other minor wording changes, and are consistent with amendments made in the parent case. Support for these amendments is present surrounding the locations where the amendments have been entered, because the nature of the amendments are clear from the context of the application.

In the present amendment, the Applicant cancels all claims 1-8, and adds new claims 9-10 to the application. Thus, upon entering these amendments, claims 9-10 will be pending in the application. Claims 9-10 were subject to restriction in the parent application and canceled at the time of allowance.

The Applicant does not intend by these claim amendments or any other amendments to abandon the subject matter of any claim as originally filed or as later presented. The Applicant reserves the right to pursue such subject matter in subsequent applications, such as continuations, CIP's, and divisionals.

The amendments include no new matter.

Respectfully submitted,

MARSHALL,GERSTEIN & BORUN
6300 Sears Tower
233 S. Wacker Drive
Chicago, Illinois 60606
Telephone: (312) 474-6300

David A. Gass
Registration No. 38,153

Date: November 8, 2001

APPENDIX OF CHANGES IN THE SPECIFICATION

At page 1, line 1:

This application is a divisional application of U.S. patent application Serial No: 09/029,156, filed August 3, 1998, which is 35 U.S.C. §371 national filing of PCT/JP96/00173, filed January 25, 1996, which in turn is a continuation-in-part of PCT/JP95/02035, filed 02 October 1995, now U.S. Patent No. 6,110,708

At page 2, lines 19-25:

Similar methods had also been tried by using another expression vectors, but the same or less expression level had merely detected by any of the vectors. Anyway, an effective expression system have [not been realized yet] not yet been realized in the art. This seems due to difficulties in expressing the conglutinin because *Escherichia coli* does not possess proteins of the structure like collagen-like region. Further, yield of the conglutinin produced from an eukaryotic cells is little, and some of the conglutinin may sometimes have an inappropriate post-transcriptional modification.

At page 4, lines 9-10:

Figure 8 shows conglutination activities on the recombinant conglutinin and the native conglutinin with[mictotier] microtiter plate assay system;

At page 5, lines 21-25:

PCR products of Example 1(1) were digested with the restriction enzymes XhoI and EcoRI, then were inserted into the expression vector pRSET-A (Invitrogen) with DNA ligation kit (Takara Shuzo). [Ligation solution was then] Then, pRSET vector so prepared from pRSET-A containing bovine conglutinin cDNA fragment was transfected into *Escherichia coli* JM109 and transformants [was] were obtained that have the conglutinin DNA fragments corresponding to 631 bp through 1113 bp of the native conglutinin DNA (Figure 1).

At page 5, line 26, to page 6, line 2:

Sequences of these fragments were corresponding to 191st through 351st amino acids of native conglutinin, namely, PCR exactly amplified the sequences having the short collagen region, the neck region and the carbohydrate recognition domain. Further, there was no error in the PCR reaction. Accordingly, [desirous] desirable stable transformants were obtained which can remarkably produce such conglutinin DNA fragments.

At page 10, lines 1-9:

After coating the microtiter plates with yeast mannan (1 μg/well), the recombinant conglutinins were reacted with sugars. Sugar binding specificity (I_{50}) was shown as sugar concentration to halve binding activities. Results are shown in Table 1. Obviously from Table 1, sugar binding activities with the recombinant conglutinin are substantially same to that of the native conglutinin. Then, as shown in Figures 6 and 7, like the native conglutinin, binding activities of the recombinant conglutinin were [depended] dependent on calcium ion. Further, these binding activities were inhibited by N-acetylglucosamine. On the other hand, tags of histidine fused to the recombinant conglutinin were not involved in the binding activities to mannan and binding specificities.

At page 11, lines 25-30:

Conglutination activities [by] of the recombinant conglutinin and the native conglutinin were evaluated by Microtiter plate assay system. Sheep erythrocyte cells with iC3b were prepared according to the method of Wakamiya *et al.*, (*Biochem. Biophys. Res. Comm.*, Vol. 187, pp. 1270-1278, 1992). Namely, 1 % sheep erythrocyte cells with iC3b were prepared by priming with a mixture of ten-fold diluted fresh horse serum and equivalent amount of anti-Forssmann antibody, and incubated at 37 °C for ten minutes.

At page 12, lines 1-10:

50 μl of 1 % sheep erythrocyte cells with iC3b and 50 μl of the recombinant conglutinin or 50 μl of the native conglutinin [was] were added to the raw veronal buffer or the veronal buffer containing 30 mM N-acetylglucosamine. Then, they were incubated at 37 °C and the conglutination activities thereon were detected. The lowest concentration of the proteins to cause agglutination is regarded as titer of conglutination, then the results are shown in Figure 8. In Figure 8, Lane A is the native conglutinin, Lane B is the recombinant conglutinin and Lane C is the recombinant conglutinin containing 30 mM N-acetylglucosamine. Titer of conglutination on the native conglutinin was 0.16 μg/ml, while that of the recombinant conglutinin was 1.3-2.5 μg/ml. Such activities were completely inhibited by 30 mM N-acetylglucosamine (GlcNAc).

At page 12, line22, to page 13, line 4:

In accordance with the method of Okuno *et al.*, (*J. Clin. Microbiol.*, Vol. 28, pp. 1308-1313, 1990), experiments were performed [by] in 96-well microtiter plates with 1 % chick's erythrocytes. The ether-treated virus antigens from [an] a hen egg antigen was used. No additive had been added to mixed cultivation solution of TBS/C (TBS solution containing 5 mM sodium chloride) except for 30 mM N-acetylglucosamine or 10 mM EDTA. After incubation at room temperature for one hour, effects on the recombinant conglutinin fragments (rBKg-CRD) against viral hemagglutination on chick's erythrocytes were observed. Results are shown in Table 2. Results on Influenza A virus A/Ibaraki/1/90 are shown in Figure 9. In Figure 9, Lane A is the native conglutinin, and Lanes B, C and D are directed to the recombinant conglutinin fragments, in which the Lane B is no additives, Lane C is added thereto 30 mM N-acetylglucosamine and Lane D is added threreto 10 mM EDTA.

At page 13, lines 18-22:

Hemagglutination Inhibition (HI) activities were depended on dosages and calcium. Further, Hemagglutination Inhibition (HI) activities of the recombinant conglutinin [is] are substantially the same level to the titer of the native conglutinin, rat surfactant protein D, human surfactant protein D (Hartshorn *et al.*, *J. Clin. Invest.*, Vol. 94, pp. 311-319, 1994).

At page 15, lines 19-25, and substitute the following paragraph:

Physiological activities against Influenza A viruses were evaluated in accordance with the evaluation method on Hemagglutination Inhibition (HI) Activities according to Example 5, the evaluation method on Neutralization Activities according to Example 6, the evaluation on Hemagglutinin (HA) Activities by Western blotting, and the present method referred to in Example 7. Further, the neutralization [activitiesagainst] activities against Influenza A viruses by the various collectins were also evaluated by the method referred to in Example 6. Results were shown in the following Tables 4 and 5.

APPENDIX OF CURRENTLY PENDING CLAIMS UPON ENTRY OF THIS AMENDMENT

Claims:

9. A purified Mannan-binding protein (MBP) having budding inhibition activity as determined by a method comprising steps of:

(a) culturing cells in presence of virus(es) to prepare virus(es)-infected cells,

(b) presenting the virus(es)-infected cells in presence or absence of the MBP,

(c) comparing gross area of virus(es)-infected focus formed in the presence of the MBP with that formed in the absence of the MBP, and

(d) evaluating from the comparison results in the step (c) an inhibition level by the MBP on budding of virus in said infected cells.

10. A purified Human Mannan-binding protein (hMBP) having anti-Influenza A virus activity as determined by the method comprising the steps of:

(a) culturing cells in presence of virus(es) to prepare virus(es)-infected cells,

(b) presenting the virus(es)-infected cells in presence or absence of a calcium-dependent lectin,

(c) comparing gross area of virus(es)-infected focus formed in the presence of a calcium-dependent lectin with that formed in the absence of a calcium-dependent lectin, and

(d) evaluating from the comparison results in the step (c) an inhibition level by the calcium-dependent lectin on budding of virus in said infected cells,

wherein the calcium-dependent lectin comprises an N-terminal region containing cysteine, a collagen-like region, a neck region and a carbohydrate recognition domain.